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CED-12/ELMO, a Novel Member of the CrkII/Dock180/Rac Pathway, Is Required for Phagocytosis and Cell Migration

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Summary

The *C. elegans* genes *ced-2*, *ced-5*, and *ced-10*, and their mammalian homologs *crkII*, *dock180*, and *rac1*, mediate cytoskeletal rearrangements during phagocytosis of apoptotic cells and cell motility. Here, we describe an additional member of this signaling pathway, *ced-12*, and its mammalian homologs, *elmo1* and *elmo2*. In *C. elegans*, CED-12 is required for engulfment of dying cells and for cell migrations. In mammalian cells, ELMO1 functionally cooperates with CrkII and Dock180 to promote phagocytosis and cell shape changes. CED-12/ELMO-1 binds directly to CED-5/Dock180; this evolutionarily conserved complex stimulates a Rac-GEF, leading to Rac1 activation and cytoskeletal rearrangements. These studies identify CED-12/ELMO as an upstream regulator of Rac1 that affects engulfment and cell migration from *C. elegans* to mammals.

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Introduction

The engulfment and removal of cells undergoing apoptosis is the final step, and perhaps the ultimate objective, of the apoptotic program (Hengartner, 2001; Savill and Fadok, 2000). Genetic screens in the nematode *Caenorhabditis elegans* have identified over a dozen genes that function in the apoptotic program (Hengartner, 1999). Seven of these genes—*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10*, and *ced-12*—are required for the efficient engulfment of apoptotic cells (Chung et al., 2000; Ellis et al., 1991; Hedgecock et al., 1983). In worms that are mutant for any of these seven genes, many cell corpses remain unengulfed for hours or even days.

Double mutant analyses have placed six of these engulfment genes into two partially redundant functional pathways: *ced-1*, *ced-6*, and *ced-7* in one, and *ced-2*, *ced-5*, and *ced-10* in the other (Chung et al., 2000; Ellis et al., 1991). Recently, these six genes have been cloned in the worm, and their homologs identified in higher organisms. Within the first group, the *ced-1* encodes a transmembrane protein (with homology to mammalian scavenger receptors) (Zhou et al., 2001), *ced-7* encodes an ATP binding cassette transporter (with homology to mammalian ABC1 and ABCR) (Luciani and Chimini, 1996; Wu and Horvitz, 1998a), and *ced-6* encodes a candidate adaptor protein (Liu and Hengartner, 1998).

In the second group of genes, *ced-2* encodes a protein homologous to the mammalian adaptor protein CrkII, *ced-5* encodes the homolog of the mammalian Dock180, and *ced-10* encodes a homolog of the Rac1 GTPase (Reddien and Horvitz, 2000; Wu and Horvitz, 1998b). The CED-2/CrkII protein is composed of one Src-homology 2 (SH2) and two Src-homology 3 (SH3) domains. CrkII was originally identified as part of a viral oncogene, but has since been implicated in a variety of cell shape and cytoskeletal changes (Kiyokawa et al., 1997; Mayer et al., 1988). Dock180 was identified as a protein that binds to the first SH3 domain of CrkII, and expression of a membrane-targeted Dock180 led to cytoskeletal changes in NIH3T3 cells (Hasegawa et al., 1996). Biochemical studies in mammalian cells and genetic studies in *Drosophila* suggest that Dock180 and its homologs function upstream of Rac activation (Erickson et al., 1997; Kiyokawa et al., 1998a; Nolan et al., 1998). The Rac GTPase has been implicated in actin polymerization at the edges of the cell, the formation of lamellipodia, and cell motility, and in Fc receptor-mediated phagocytosis (Bishop and Hall, 2000; Caron and Hall, 1998; Massol et al., 1998; Nobes and Hall, 1999; Van Aelst and D'Souza-Schorey, 1997). Genetic studies in the worm and recent studies in mammalian cells suggest that CED-10/Rac functions downstream of CED-2/CrkII and CED-5/Dock180 during engulfment of apoptotic cells (Albert et al., 2000; Leverrier and Ridley, 2001; Reddien and Horvitz, 2000; Tosello-Trampont et al., 2001). Thus, the products of the genes in the second complementation group appear to regulate reorganization of the cytoskeleton during engulfment (Chimini and Chavrier, 2000).

Table 1. *ced-12* Is Required for Proper Gonad Development and Distal Tip Cell Migration in Hermaphrodites

	Hermaphrodite						Male
	Anterior Gonad Defects (%)			Posterior Gonad Defects (%)			Defects (%)
Genotype	Extra Turn	Branching	Other	Extra Turn	Branching	Other	
ced-12(bz187)	9 ± 3	4 ± 2	5 ± 2	44 ± 5	13 ± 3	8 ± 3	0
ced-12(k145)	15 ± 4	0	3 ± 2	31 ± 5	7 ± 3	8 ± 3	1 ± 1
ced-12(k149)	13 ± 3	3 ± 2	6 ± 2	34 ± 4	23 ± 3	8 ± 2	1 ± 1
ced-12(k156)	18 ± 3	0	6 ± 2	26 ± 3	8 ± 2	4 ± 2	1 ± 1
ced-12(k158)	54 ± 4	0	11 ± 2	44 ± 4	2 ± 1	16 ± 3	0
ced-12(oz167)	29 ± 4	0	8 ± 2	42 ± 4	0	8 ± 2	4 ± 2

Percentages of misshapen hermaphrodite and male gonads in late L4 or young adult animals grown at 20°C were examined. Males used were of *him-5(e1490)* background, except for *bz187* males, which were scored in a wild-type background. *him-5(e1490)* males had a 1% DTC migration defect. n = 160 for *k145*, *k149*, *k156*, and *k158* hermaphrodites, and 100 for *bz187* and *oz167* hermaphrodites and all males. Extra turn: extra-turning on dorsal muscle (see Figures 1E and 1F); branching: branching occurred at or after second turn, and resulted in an extra small "appendage" (see Figure 1E); other: all other visible defects, e.g. meandering or premature stop on dorsal muscle. All data shown are averages ± SEM.

Worms with mutations in *ced-2*, *ced-5*, or *ced-10* also show a second defect: the abnormal migration of the distal tip cells (DTC) (Reddien and Horvitz, 2000; Wu and Horvitz, 1998a). Interestingly, in mammalian cells, CrkII, Dock180, and Rac1, along with another adaptor protein p130Cas, have also been linked to cell migration, invasion of tumor cells, and cell survival (Cheresh et al., 1999; Klemke et al., 1998). Thus, Rac1 and its upstream activators likely play a key role in cellular migration during development.

In this report, we present evidence that *ced-12*, and its mammalian orthologs *elmo1* and *elmo2*, encode new members of the CrkII/Dock180/Rac pathway. In *C. elegans*, CED-12 is required for the efficient engulfment of dying cells and is also essential for cell migration. In mammalian cells, ELMO1 functionally cooperates with CrkII and Dock180 during phagocytosis and functions upstream of Rac1. These studies place *ced-12/elmo* as an important member of the *ced-2/ced-5/ced-10* pathway that regulates engulfment and cell migration in *C. elegans* and mammals.

Results

All six *ced-12* alleles described here share a similar set of defects, which include a deficiency in the removal of apoptotic cell corpses, defects in migration and gonadal morphology in the hermaphrodite, and a partially penetrant embryonic lethality (Tables 1–3; Figure 1). These defects are described in more detail below.

ced-12 Is Required for the Removal of Dying Cells

Removal of apoptotic cells is a rapid process in *C. elegans*: in the wild-type, dying cells are engulfed within an hour of onset of death (Sulston and Horvitz, 1977). In contrast, both somatic and germ cell corpses persist in *ced-12* mutants, leading to an accumulation of dead cell corpses (Figures 1A and 1B; Supplemental Table S1, see Supplemental Data, below). This persistent cell corpse phenotype was abrogated if cell death was prevented, e.g., through inactivation of the *ced-3* caspase homolog (Supplemental Table S1). In addition to its role in the engulfment of apoptotic corpses, *ced-12* is also required for the efficient removal of cells that die by necrosis-like processes (Chung et al., 2000), suggesting that its function is not restricted to the apoptotic program.

ced-12 Is Required for Many Developmental Processes

Additional phenotypic analysis revealed that *ced-12* is required for a large number of developmental processes. Most dramatically, approximately one-fifth of the *ced-12(oz167)* mutant embryos we analyzed suffered from abnormal development, usually with lethal consequences (Supplemental Table S1). The range of defects was broad, with some embryos failing to initiate proper morphogenesis and others aborting at various stages during the elongation process. The wide range of terminal phenotypes would be consistent with a general, but low, penetrance failure in cell migration. Mutations in *ced-12* also cause sublethal developmental defects,

Table 2. *elmo* Expression Partially Rescues DTC Migration Defect in *ced-12* Mutants

Genotype	Anterior Gonad Defects (%)	Posterior Gonad Defects (%)	n
ced-12(oz167)	29 ± 5	42 ± 5	100
ced-12(oz167); opEx478[sur-5::GFP]	16 ± 4	43 ± 6	81
ced-12(oz167); opEx458[eft-3::ced-12]	4 ± 2	9 ± 3	92
ced-12(oz167); opEx481[eft-3:: elmo1-FLAG]	6 ± 3	15 ± 4	83
ced-12(oz167); opEx475[eft-3:: elmo2-FLAG]	5 ± 2	16 ± 4	81

Extra turn defects in anterior and posterior gonad of animals transgenic for *ced-12* or tagged *elmo*-expressing cDNAs. All extrachromosomal arrays are marked with *sur-5::GFP*. n, number of animals analyzed. All data shown are averages ± SEM.

Table 3. *ced-12* Acts in Engulfing Cells Upstream of CED-10/Rac

Parental Genotype	Transgenic	Heat Shock	Cell Corpses	n
A. Overexpression of a *ced-12* cDNA rescues the *ced-12* engulfment defect				
ced-12(oz167)	N/A	−	7.3 ± 1.0	64
ced-12(oz167)	N/A	+	8.0 ± 1.6	42
ced-12(oz167); opEx434[hs::ced-12]	−	−	5.7 ± 1.0	42
ced-12(oz167); opEx434[hs::ced-12]	+	−	2.7 ± 1.1	11
ced-12(oz167); opEx434[hs::ced-12]	−	+	7.2 ± 1.6	36
ced-12(oz167); opEx434[hs::ced-12]	+	+	0.3 ± 0.3	21
ced-12(oz167); opEx435[hs::ced-12]	−	−	7.3 ± 2.2	16
ced-12(oz167); opEx435[hs::ced-12]	+	−	3.2 ± 0.9	22
ced-12(oz167); opEx435[hs::ced-12]	−	+	9.1 ± 2.4	20
ced-12(oz167); opEx435[hs::ced-12]	+	+	0.1 ± 0.1	27
ced-12(oz167); opEx431[hs::gfp]	−	+	13.3 ± 1.9	8
ced-12(oz167); opEx431[hs::gfp]	+	+	10.9 ± 1.0	20
B. Overexpression of *ced-12* promotes the engulfment of persistent cell corpses				
ced-12(oz167); opEx434[hs::ced-12]	−	+	8.8 ± 0.7	31
ced-12(oz167); opEx434[hs::ced-12]	+	+	0.7 ± 0.2	33
C. Overexpression of *ced-12* does not rescue other engulfment-defective mutants				
ced-1(e1735); opEx435[hs::ced-12]	−	+	14.8 ± 1.6	11
ced-1(e1735); opEx435[hs::ced-12]	+	+	16.8 ± 0.7	32
ced-6(n1813); opEx434[hs::ced-12]	−	+	8.4 ± 1.1	12
ced-6(n1813); opEx434[hs::ced-12]	+	+	10.0 ± 0.8	34
ced-7(n1892); opEx435[hs::ced-12]	−	+	21.3 ± 2.1	12
ced-7(n1892); opEx435[hs::ced-12]	+	+	19.0 ± 1.3	12
ced-2(e1752); opEx434[hs::ced-12]	−	+	10.5 ± 1.0	12
ced-2(e1752); opEx434[hs::ced-12]	+	+	13.6 ± 1.1	28
ced-5(n1812); opEx435[hs::ced-12]	−	+	22.5 ± 1.6	13
ced-5(n1812); opEx435[hs::ced-12]	+	+	21.6 ± 2.6	11
ced-10(n1993); opEx435[hs::ced-12]	−	+	9.6 ± 1.2	13
ced-10(n1993); opEx435[hs::ced-12]	+	+	9.0 ± 0.9	20
D. Overexpression of *ced-10* rescues the engulfment defect of *ced-12* mutants				
ced-10(n1993); opEx700[hs::ced-10]	+	−	12.0 ± 3.1	7
ced-10(n1993); opEx700[hs::ced-10]	+	+	0.3 ± 0.1	23
ced-12(oz167); opEx700[hs::ced-10]	−	−	12.4 ± 1.5	9
ced-12(oz167); opEx700[hs::ced-10]	−	+	13.5 ± 1.8	10
ced-12(oz167); opEx700[hs::ced-10]	+	−	19.4 ± 3.4	11
ced-12(oz167); opEx700[hs::ced-10]	+	+	0.6 ± 0.2	23

Transgenic and nontransgenic progeny from transgenic parents were given a heat shock pulse as embryos (A, C, and D) or L1 larvae (B), and the number of persistent cell corpses in the head were scored 12 hours later. Data shown are average ± SEM. Two independent transgenic extrachromosomal arrays (*opEx434* and *opEx435*) expressing a full-length *ced-12* cDNA under the control of the heat-shock promoters *hsp16-41* and *hsp-16.2* were tested. As a negative control, a transgenic array (*opEx431*) expressing GFP under the same promoter combination was used. n, number of animals scored. N/A, not applicable. Note that, in section A, transgenic animals without heat-shock also show a partial rescue, likely due to leakiness of the promoter in the arrays tested. A P_{eft-3}::*gfp* plasmid was used as a coinjection marker for Figures 3A–3C; for Figure 3D, P_{sur-5}::*gfp* was used.

including small embryos, delayed development, and reduced fertility (Supplemental Table S1).

ced-12 Is Required for DTC Migration and Gonadal Morphogenesis

The distal tip cells guide the developing hermaphrodite gonad into a bilobed U-shaped form (Figure 1D) (Hubbard and Greenstein, 2000). In *ced-12(lf)* hermaphrodites, migration of the distal tip cells was frequently misdirected, resulting in gonads with abnormal morphology (Figures 1E and 1F; Tables 1–3). In addition, gonad arms often had an uneven diameter, with a very distended proximal end and/or small branches or extrusions at bends (Figure 1E, Table 3A). In contrast to hermaphrodites, gonads were overtly normal in *ced-12* males (Table 1). However, despite their normal gonad morphology, *ced-12* males have difficulty in siring progeny (data not shown), suggesting that *ced-12* is required for at least one other aspect of male mating. The embryonic lethality and the diverse cell migration defects were still present in *ced-12(lf); ced-3(lf)* double mutants (Supplemental Table S1, see Supplemental Data, below), confirming that CED-12 acts in these processes independently of its role in engulfment.

ced-12 Acts in the *ced-2/ced-5/ced-10* Pathway

Genetic studies suggest that the engulfment *ced* genes act in two partially redundant, parallel pathways, with

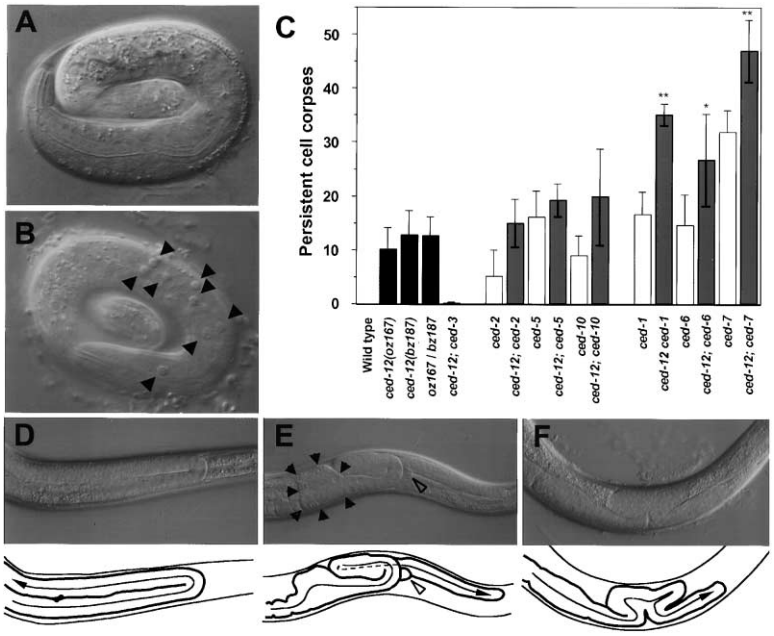


Figure 1. *ced-12* Is Required for the Engulfment of Apoptotic Cell Corpses and Gonadal Cell Migration

(A and B) Wild-type (A) and *ced-12(oz167)* (B) late embryos (three-fold stage). By this stage of development, all embryonic cell deaths have been cleared in the wild-type embryo, whereas the *ced-12* embryo contains many persistent cell corpses (arrowheads).

(C) *ced-12* belongs to the *ced-2/ced-5/ced-10* redundancy group. Animals were scored for persistent cell corpses in the heads of young L1 larvae. Black bars, *ced-12* single mutants; open bars, single engulfment mutants; gray bars, double mutant pairs for *ced-12(oz167)* and the other engulfment genes. Data shown are means ± standard deviation. *, $p < 0.01$; ** $p < 0.001$ for double mutant versus the stronger of the respective single mutants.

(D–F) Distal tip cell (DTC) migration defect. In wild-type hermaphrodites, the DTC follows a stereotyped migration pattern resulting by the late L4 stage in a U-shaped gonad (D). Variable migration defects in *ced-12* mutants result in twisted gonads that often fail to reflex or make extra turns (E) and (F). *ced-12* mutants also occasionally show a distended proximal tube (filled arrowheads in E) as well as short branching or distensions (open arrowhead in E). Deduced DTC migration path is indicated by gray arrow in camera lucida drawing below each Normarski picture. Anterior is to the left, dorsal to the top.

ced-1, *ced-6*, and *ced-7* acting in one pathway, and *ced-2*, *ced-5*, and *ced-10* in the other (Ellis et al., 1991). To determine to which pathway *ced-12* belongs, we determined the extent of persistent cell corpses in double mutants between *ced-12* and the other engulfment genes (Figure 1C). Double mutants between *ced-12* and *ced-1*, *ced-6*, or *ced-7* exhibited significantly more cell corpses than the respective single mutants. In contrast, we observed only a modest increase in *ced-12*; *ced-2*, *ced-12;ced-5*, and *ced-12*; *ced-10* double mutants. These data suggest that *ced-12* acts in the same genetic pathway as *ced-2*, *ced-5*, and *ced-10*. Consistent with this categorization, DTC migration defects are only observed in *ced-2*, *ced-5*, *ced-10*, and *ced-12* mutants, but not in *ced-1*, *ced-6*, or *ced-7* mutants (Reddien and Horvitz, 2000; Wu and Horvitz, 1998b).

Cloning of *ced-12*

ced-12 had previously been mapped to chromosome *I* close to and to the left of *lin-11* (Chung et al., 2000). We rescued *ced-12(oz167)* animals with the yeast artificial chromosomes Y45G3, Y49H10, and Y39F7, after our attempts to rescue with cosmids spanning this region were unsuccessful (Supplemental Figure S1B, see Supplemental Data, below). Using a set of single nucleotide polymorphisms (SNPs, see Experimental Procedures), we narrowed *ced-12* to one of three genes in the cosmid gap covered by Y106G6E (Supplemental Figure S1C). Two lines of evidence suggested that *ced-12* corresponds to Y106G6E.5. First, we could identify nonsense mutations in Y106G6E.5 in all six *ced-12* mutant alleles that we analyzed, resulting in premature termination of translation (Supplemental Figure S1D, and Figure 2A). Second, expression of a full-length Y106G6E.5 cDNA fully rescued the engulfment and DTC migration defect of *ced-12* mutants (Tables 1–3).

We determined the intron-exon structure of *ced-12* by sequencing six independent cDNAs and by RT-PCR amplification on *ced-12(+)* mRNA. *ced-12* mRNA can be *trans*-spliced to both SL1 or SL2 splice leaders (data not shown). There were no splice variations among the six sequenced cDNAs. Northern blot analysis of polyA$^+$ mRNA of wild-type and two *ced-12* mutant animals showed a single band of 2.4 kb (Supplemental Figure S1E). *ced-12* message levels appeared reduced in *ced-12(oz167)* and *ced-12(bz187)* mutants, likely due to nonsense-mediated decay (Hilleren and Parker, 1999).

ced-12 encodes a previously uncharacterized protein of 731 amino acids (Figure 2A) whose molecular function is not obvious from the sequence analysis. However, *ced-12* is clearly conserved through evolution, possessing at least one homolog in *Drosophila* (previously uncharacterized gene CG5336), and three in mice and humans (see below, Figure 2A).

ced-12 Promotes the Engulfment of Apoptotic Cells and Is Required in the Engulfing Cell

To conclusively demonstrate that Y106G6E.5 corresponds to *ced-12*, we generated transgenic *ced-12(lf)* worms expressing a full-length Y106G6E.5 cDNA under the control of the *C. elegans* heat shock promoters *hsp-16.2* and *hsp-16.48*. Together, these promoters drive expression in almost all somatic cells upon heat shock stimulus (Stringham et al., 1992), including the cells fated to die (Hengartner and Horvitz, 1994) and engulfing cells (Robertson and Thomson, 1982). Overexpression of Y106GE.5 cDNA just before the major wave of embryonic cell death almost completely eliminated the persis-

tence of cell corpses in the heads of young transgenic *ced-12(lf)* larvae (Table 3A).

To determine whether *ced-12* acts in the dying or the engulfing cells, we expressed *ced-12* in L1 and L2 larvae (12–24 hr after the wave of embryonic cell death). Previous work has shown that the *hsp-16.2* and *hsp-16.48* heat shock promoters cannot direct new gene expression in late apoptotic corpses (Liu and Hengartner, 1998; Reddien and Horvitz, 2000). Rescue, therefore, can only occur if the expression of *ced-12* in living, engulfing cells was sufficient for phagocytosis of cell corpses. The number of persistent corpses was dramatically reduced when *ced-12* expression was induced in larvae (Table 3B and data not shown), indicating that *ced-12* expression in the engulfing cell is sufficient for engulfment.

ced-12 May Act Upstream of *ced-10*

We also tested whether overexpressed CED-12 could compensate for the loss of any of the other engulfment genes. This method has previously been used to show that *ced-6* acts downstream of *ced-1* and *ced-7*, and that *ced-10* acts downstream of *ced-2* and *ced-5* (Liu and Hengartner, 1998; Reddien and Horvitz, 2000). We discovered that overexpression of *ced-12(+)* did not significantly rescue of any of the other mutants tested (Table 3C). In contrast, overexpression of *ced-10/rac* efficiently rescued *ced-12* mutants (Table 3D). These results suggest that CED-12, like CED-2 and CED-5, functions as an upstream activator of CED-10 Rac.

Cloning of Mammalian Orthologs of *ced-12*

Searching the DNA and protein databases identified multiple human and murine expressed sequence tags (ESTs) with homology to CED-12. We then cloned the full-length human *ced-12* homolog from a human macrophage library using a PCR strategy (see Experimental Procedures). This human *ced-12* gene was predicted to encode a 727 amino acid polypeptide with 44% similarity to *C. elegans* CED-12. Analysis of the human genome sequence database revealed that in addition to this *ced-12* gene located on chromosome 7, there was a second homologous gene located on chromosome 20. A human EST clone (BEO17955) that corresponded to this second *ced-12* gene provided the sequence of a 722 aa polypeptide. Two murine ESTs (AI574349 and AA711524) provided the sequence of the corresponding mouse *ced-12* genes. We have named the mammalian *ced-12* orthologs as *elmo* (genes involved in engulfment and cell motility), and refer to the products of the two *ced-12* genes as ELMO1 and ELMO2. The identified sequences appeared to encode the full-length open reading frame for both *elmo1* and *elmo2* genes of human and mouse based on several criteria (see Supplemental Experimental Procedures, Supplemental Data, below). More recent genome and EST analyses have identified a third *elmo* gene (located on chromosome 16, EST clones NM024712; AK023886); however, the complete sequence of the *elmo3* gene has not yet been verified.

The mouse and human ELMO1 polypeptides are highly conserved with 98% amino acid identity, as is also the case with ELMO2 from the two species. ELMO1 had 75% identity and 88% similarity to ELMO2. In comparison, both ELMO1 and ELMO2 have about 44% similarity to the *C. elegans* CED-12 and 65% similarity to the *Drosophila* CED-12 (Figure 2A). *C. elegans* and *Drosophila* contain only one gene representing *ced-12*, while there are three family members in humans and mice.

The predicted CED-12 and ELMO proteins appear unique, with no obvious catalytic domain. Computer analysis predicted ELMO1 and ELMO2 to be soluble cytoplasmic proteins. A potential SH3 binding PxxP motif near the C terminus (conserved from worm to human), and a putative leucine zipper around amino acid 630 (that was found in all of the mammalian proteins, but not in the worm or fly proteins), were detectable. A putative pleckstrin homology (PH) domain (around amino acid 555–676 of ELMO1) was predicted in the *Drosophila* and mammalian proteins (absent in worm CED-12) by the SMART algorithm, but was not predicted by several other algorithms.

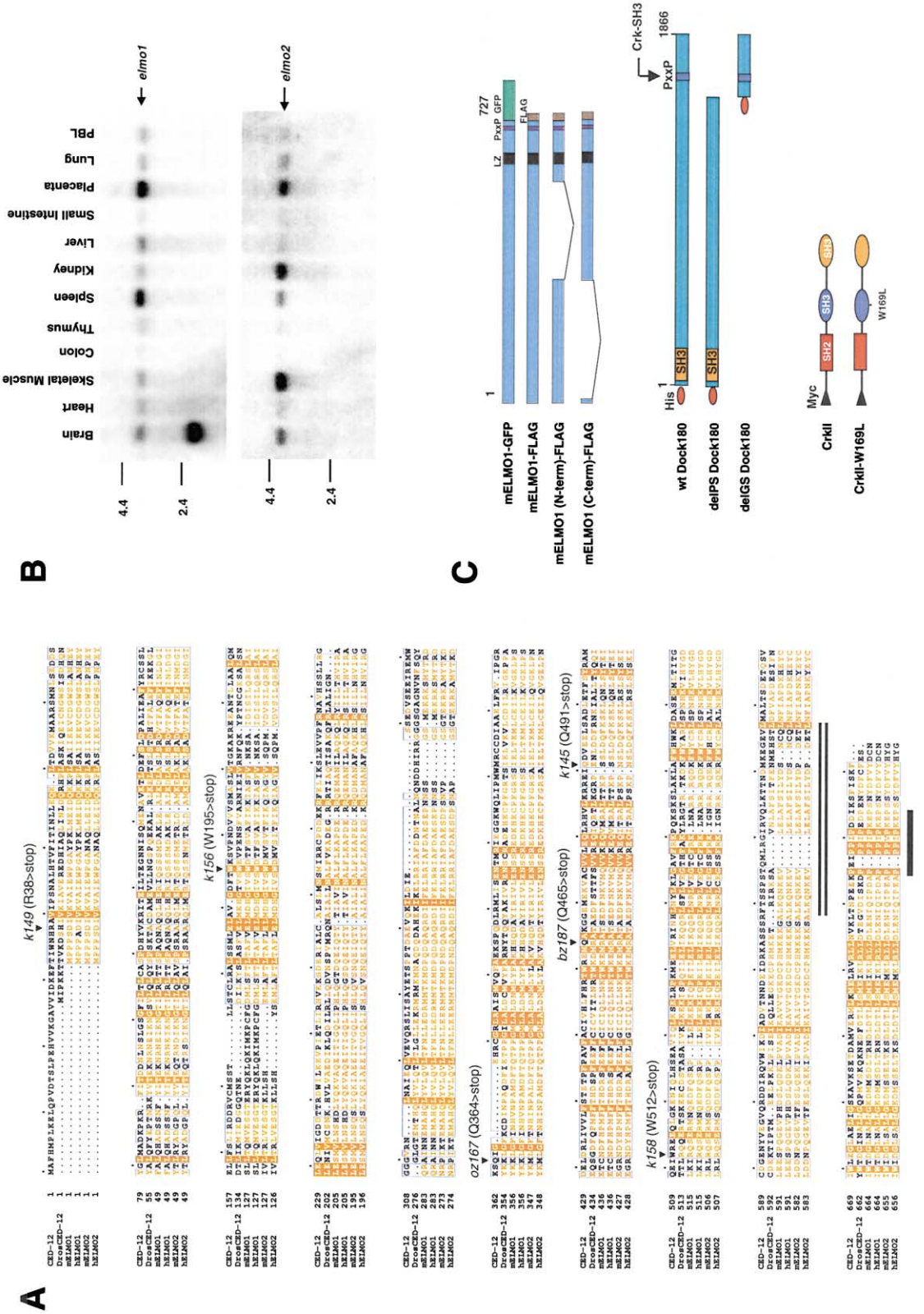
A Northern blot of polyA$^+$ RNA from various tissues using an *elmo1* probe showed that it is widely expressed in all tissues as a 4.2 kb transcript (Figure 2B). *elmo2* had a slightly different expression pattern compared to *elmo1.* The >100 ESTs that exist in the database for human and murine *elmo1* and *elmo2* originate from a large variety of tissues, confirming that both genes are widely expressed. RT-PCR using primers specific for *elmo1* or *elmo2* showed expression of both genes in the macrophage line RAW264.7, murine B cell line A20, and Jurkat T cell line, as well as in the LR73 cell line used in our subsequent experiments described below (data not shown).

Mammalian ELMO Can Partially Substitute for CED-12 to Promote DTC Migration

To test whether CED-12/ELMO might be functionally conserved, we generated transgenic worms expressing tagged ELMO1 and ELMO2 under the control of the ubiquitous promoter *eft-3*, and examined their ability to rescue the engulfment and DTC migration defects of *ced-12* mutants. While the ELMO constructs did not significantly rescue the engulfment defect (data not shown), constitutive expression of ELMO1 or ELMO2 could almost completely rescue the DTC migration defect (Table 3B), strongly suggesting that ELMO1 and ELMO2 can interact with the appropriate CED-12 partners in *C. elegans*. We do not know why ELMO did not rescue the engulfment defect of *ced-12* mutants, but Wu and Horvitz (1998b) have also reported a similar partial rescue of *ced-5* mutants by the CED-5 homolog Dock180.

ELMO1 Cooperates With CrkII and Dock180 during Engulfment

To determine whether ELMO1 or ELMO2 may play a role in phagocytosis in mammalian cells, we generated FLAG or GFP-tagged constructs of mouse ELMO1 and ELMO2 (see Figure 2C for a schematic). We transiently transfected the phagocytic fibroblast line LR73 with ELMO1-GFP, ELMO2-GFP, or as a control, GFP alone (in duplicate). Using two-color flow cytometry, we determined the fraction of GFP positive cells that could engulf "red" fluorescent 2 μm carboxylate-modified latex beads, which we have previously shown can serve as a simpli-



fied target that mimics the negatively charged apoptotic cells (Tosello-Trampont et al., 2001). Surprisingly, expression of ELMO1 or ELMO2 resulted in a reduced uptake compared to GFP alone (Figure 3A) (seen in >20 independent experiments). FLAG-tagged ELMO also gave similar results as GFP-tagged ELMO (Figure 3D and data not shown). Expression of unrelated proteins, such as the adaptor protein Shc or glutathione S-trans-

ferase (GST), did not cause inhibition of phagocytosis (data not shown). The uptake of 0.1 μm carboxylate-modified beads (indicative of pinocytosis) was unaffected by ELMO1-GFP expression (data not shown), arguing against a general inhibitory effect of ELMO on plasma membrane events. When we tested a membrane-targeted version of ELMO1 (ELMO1-CAAX), it also showed an inhibition of engulfment similar to that observed with ELMO1-FLAG (Supplemental Figure S2, see Supplemental Data, below). This suggested that forced membrane targeting of ELMO1 per se does not provide a gain of function.

Based on the genetic studies in *C. elegans* above, we predicted that in mammalian cells, ELMO1 would also function in the CrkII/Dock180/Rac pathway during engulfment. We tested whether coexpression of any of these proteins with ELMO would "rescue" the inhibition of engulfment. We tested the effect of overexpression of Dock180, either alone or with ELMO1-GFP. Transient transfection of a plasmid encoding Dock180 alone also led to a small but reproducible inhibition of uptake (Figure 3B). In contrast, cotransfection of Dock180 and ELMO1-GFP led to a roughly 2-fold increase in bead uptake compared to the GFP-alone control (mean increase of 2.36 ± 0.46, $p < 0.01$, $n = 4$). The "efficiency" of bead uptake by the Dock180+ELMO1 transfected cells was also increased, as revealed by the mean fluorescence intensity (MFI) of individual cells (roughly indicative of the number of beads taken up per cell) (Figure 3B). These data suggest a functional cooperation between Dock180 and ELMO during engulfment.

To determine which regions of Dock180 are required for cooperation with ELMO, we expressed either the delPS mutant of Dock180, which lacks the C-terminal 130 amino acids including the CrkII binding site, or the delGS mutant, which lacks most of the N terminus of the protein but is still capable of binding to CrkII (Figure 2C) (Kiyokawa et al., 1998b). Neither deletion construct cooperated with ELMO1 (Figure 3B); thus, both regions of Dock180 appeared necessary for the enhancement of engulfment.

Transfection of CrkII alone led to an enhancement of engulfment, as we have previously reported (Tosello-Trampont et al., 2001). Cotransfection of CrkII and ELMO1 led to a further enhancement of phagocytosis (mean increase of 1.23 ± 0.06, $p < 0.01$, $n = 3$), compared to transfection with CrkII alone (Figure 3C). Moreover, the "efficiency" of uptake after CrkII and ELMO1-GFP cotransfection was consistently greater than transfection with CrkII alone. These data were suggestive of a functional cooperation between ELMO1 and CrkII.

When all three plasmids encoding CrkII, Dock180, and ELMO1 were cotransfected, the efficiency of bead uptake (as determined by MFI) was consistently the highest among all conditions tested, although the percentage of engulfing cells was comparable to ELMO1+CrkII coexpression (Figure 3C). Simultaneous expression of all three proteins was required to obtain the maximal uptake, since cotransfection of CrkII and Dock180, without ELMO1, led to a lower uptake than overexpressing CrkII alone (Figure 3C). Moreover, mutants of Dock180, or an SH3 mutant of CrkII (W169L) that fails to interact with Dock180, did not show the synergy in the triple transfections (Figure 3C and data not shown). Taken together, these data suggest that ELMO1, Dock180, and CrkII can functionally cooperate during phagocytosis.

CrkII/Dock180/ELMO Proteins Function Upstream of Rac

Since CED-10/Rac is considered the most downstream component in the pathway (Reddien and Horvitz, 2000), we tested if an activated form of Rac (Rac1L61) can overcome the inhibition due to ELMO1. Expression of Rac1L61 enhanced the uptake compared to cells expressing GFP alone, and the cotransfection of ELMO1 did not affect the Rac1L61 mediated uptake (Figure 3D). The dominant negative Rac1N17 mutant led to an inhibition of uptake, which was unaffected by coexpression of ELMO1. Moreover, the dominant negative Rac1N17 inhibited the enhanced uptake due to ELMO1+Dock180 and ELMO1+Dock180+CrkII cotransfection (Figure 3E). These data suggested that ELMO1, CrkII, and Dock180 function upstream of Rac during phagocytosis, consistent with the order of gene function in *C. elegans*.

Biochemical Interaction Between ELMO and Dock180

We examined if there might be a physical interaction between ELMO1 and either Dock180, Rac, or CrkII. After transient transfection in COS-7 cells with plasmids encoding ELMO1-GFP and His-Dock180, ELMO1-GFP was specifically coprecipitated with His-Dock180 (Figure 4A). Similarly, FLAG-tagged ELMO1 specifically coprecipitated His-Dock180 only when both proteins were coexpressed (Figure 4B, lane 7). Dock180 was not coprecipitated by FLAG-tagged human CED-6 used as a control (lane 9). Among the mutants of Dock180 (see schematic in Figure 2C), the delPS mutant bound to ELMO1 (Figure 4B, lane 10), but the delGS mutant failed to interact with ELMO1 (Figure 4B, lane 11). Since the delPS mutant lacks the proline-rich motifs necessary for CrkII binding to Dock180, it appears that ELMO binding to Dock180 can occur in the absence of the CrkII-Dock180 interaction.

Crude analysis of the region of ELMO required for Dock180 binding suggested that the C-terminal region

Figure 2. Identification and Characterization of *ced-12* Homologs

(A) Alignment of the predicted protein sequence of *C. elegans* CED-12, *Drosophila* CED-12, and ELMO1 and ELMO2 from mouse and human. Invariant residues are highlighted and conserved residues are shaded. The putative SH3 binding proline-rich motif in the C terminus, present in all of the CED-12 family members, is underlined, and the putative leucine zipper motif is double underlined (seen in both ELMO1 and ELMO2 of human and mouse, but absent in the worm and fly proteins). Arrowheads above the worm protein sequence indicate mutations affecting the *C. elegans* CED-12 protein.

(B) Northern analysis of polyA$^+$ RNA from various tissues using an *elmo1* or *elmo2* probe demonstrated that both genes are widely expressed (see Supplemental Experimental Procedures).

(C) Schematic diagram of the wt and the truncated forms of ELMO1, as well as Dock180 and CrkII constructs used in this study.

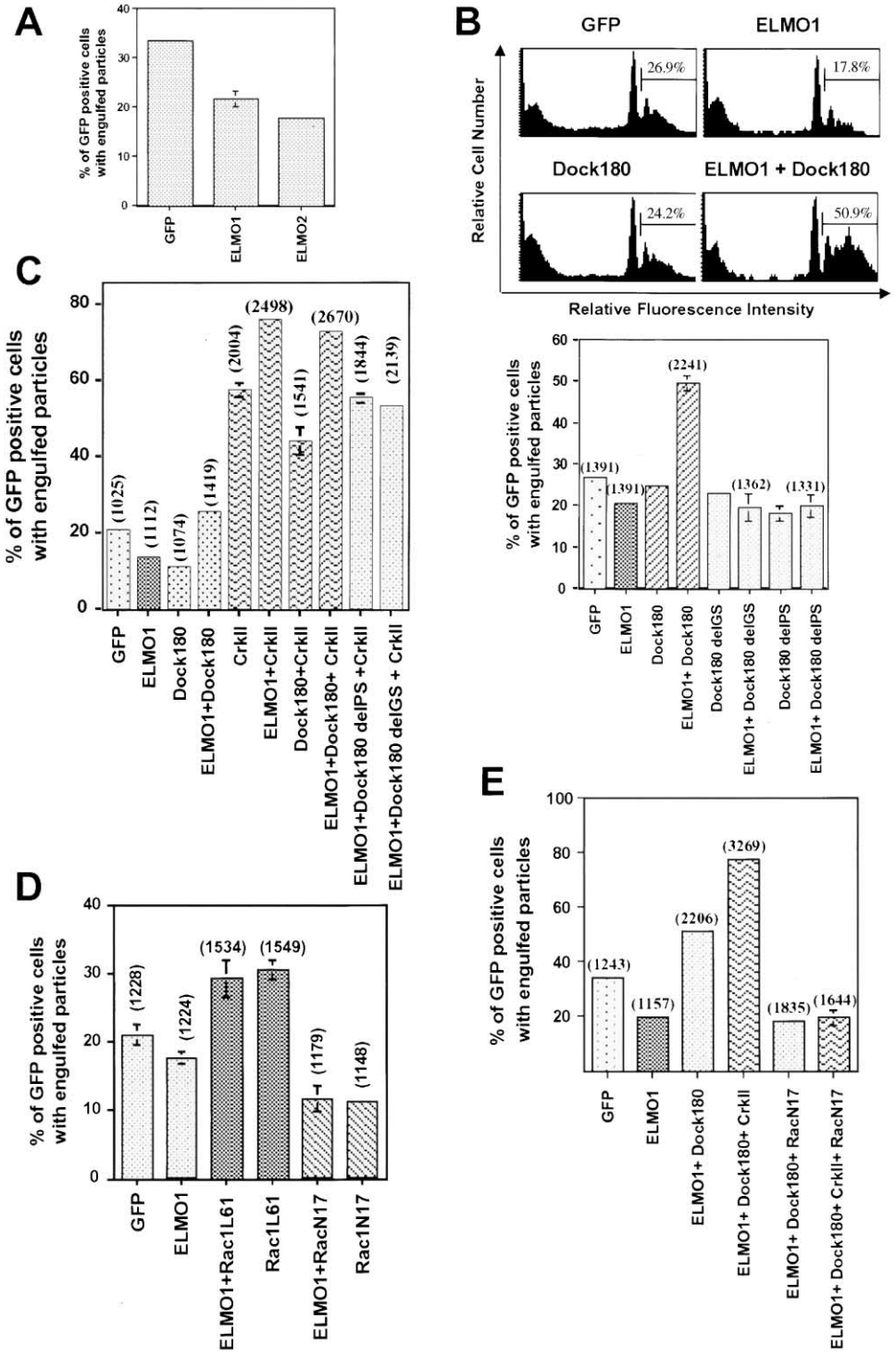


Figure 3. Functional Cooperation between ELMO1, Dock180, and CrkII During Phagocytosis

(A) Inhibition of phagocytosis by transient expression of ELMO1 or ELMO2. LR73 cells were transiently transfected in duplicate with plasmids coding for GFP, ELMO1-GFP, or ELMO2-GFP. The engulfment of "red" fluorescent 2 μm carboxylate-modified beads (30,000 cells per sample) was analyzed by two-color flow cytometry. The fraction of phagocytic GFP positive cells and the standard deviations of duplicate samples are shown.

(B) Functional cooperation between ELMO1 and Dock180. ELMO1-GFP plasmid (0.8 μg) was cotransfected with Dock180 (1.8 μg), delGS Dock180 (1.3 μg), or delPSDock180 (1.7 μg) (plasmid concentrations were altered to normalize for the differences in plasmid size). Carrier DNA (FLAG-tagged vector alone) was added to keep the same plasmid concentration in the different samples. The phagocytosis assay was performed as described above. The histogram profiles of the "green" cells for their mean fluorescence intensity (MFI), indicative of the "efficiency" of bead uptake, are also shown. We excluded the sharp first peak in the gate setting, as it reflects fluorescence due to bound

of ELMO1 (see schematic in Figure 2C) was necessary and sufficient for interaction with Dock180 (Figure 4B, lane 8, and Figure 4C, lane 4). ELMO2 also coprecipitated Dock180 (Figure 4C, lane 1), indicating that both forms of ELMO can interact with Dock180. We also tested whether the *C. elegans* CED-12 protein can interact with mammalian Dock180; however, these experiments were inconclusive, as the worm protein was poorly expressed in COS-7 or 293T cells. When we examined the interaction of the worm CED-12 and the worm CED-5 in a yeast two-hybrid assay, we were able to detect an interaction between these two proteins, indicating that the CED-12/ELMO and CED-5/Dock180 interaction is evolutionarily conserved (Figure 4D). In the same two-hybrid assay, we did not detect an interaction between wt or mutant forms of Rac and CED-12 (Figure 4D).

When we tested whether ELMO1 can interact with CrkII, we failed to detect CrkII in ELMO1 immunoprecipitates (Figure 4E, lane 5), while Dock180 was readily coprecipitated with ELMO1 (Figure 4E, lane 6). Crk immunoprecipitates also failed to coprecipitate ELMO1 (data not shown). Since Dock180 can interact with both ELMO1 and CrkII, we tested whether a trimeric complex of ELMO1/Dock180/CrkII could be formed. Indeed, cotransfection of all three genes resulted in the coprecipitation of CrkII with ELMO1 (Figure 4E, lane 7). The trimeric complex was not observed when the delPS or delGS mutants of Dock180 were coexpressed (data not shown). These data suggested that ELMO1, Dock180, and CrkII could form a trimeric complex, most likely through Dock180 bridging ELMO1 and CrkII. The biochemical complex between ELMO1/Dock180/CrkII appears to be functionally relevant, since LR73 cells transfected with all three plasmids showed the highest efficiency of uptake, and mutants of Dock180 or CrkII that fail to interact with each other failed to cooperate during engulfment (Figure 3).

ELMO1, Together with CrkII and Dock180, Induces Cytoskeletal Changes and Localizes to Membrane Ruffles

Rac is essential for actin polymerization at the leading edges of the cell and is thought to facilitate cell migration through the protrusion of lamellipodia (Nobes and Hall, 1999). Since CED-12/ELMO functioned upstream of Rac during phagocytosis and cell migration, we asked if ELMO1 could promote actin polymerization. We transfected LR73 cells with the indicated plasmids and analyzed the localization of ELMO1 through its GFP fluorescence and the F-actin through rhodamine-labeled phalloidin (Figure 5). LR73 cells normally have a spindle-like shape; this shape was not affected by expression of GFP or Dock180 alone (Figures 5a and 5c). In contrast, expression of ELMO1-GFP alone, which localized primarily in the cytoplasm (Figure 5b), caused about two-thirds of the cells to adopt an extended/polygonal shape (based on counting of GFP positive and GFP negative cells in multiple fields—see Supplemental Figure S3, Supplemental Data, below). Cells transfected with Dock180 and ELMO1-GFP were quite similar in shape to cells expressing ELMO1-GFP alone; however, we could observe a minor, but detectable, colocalization of ELMO1-GFP with F-actin in these doubly transfected cells (revealed as yellow in the overlay; Figure 5f), and some localization of ELMO1-GFP to the plasma membrane. When CrkII alone was coexpressed with ELMO1, there was ruffling of the membrane, and some localization of ELMO1 to the membrane that was also detectable (Figure 5g). However, a striking change in cell shape, along with relocalization of ELMO1-GFP to abundant membrane ruffles, was observed when CrkII, ELMO1, and Dock180 were cotransfected (Figure 5h).

Since ruffling is a Rac1-dependent event (Ridley et al., 1992), we tested the effect of coexpressing dominant negative Rac1. The ruffling due to expression of ELMO1/Dock180/CrkII was nearly lost when Rac1N17 was cotransfected (Figure 5i). Despite the overall diminished staining for F-actin due to Rac1N17 coexpression, we could still detect the localization of ELMO1-GFP to the membranes (seen in multiple cells in the field). This is consistent with a model that Rac1 functions downstream of ELMO1, in which case Rac1N17 would not be expected to affect the ELMO1 localization per se. In these experiments, we did not see an effect on actin bundling or stress fibers due to ELMO1-GFP or CED-12-GFP expression in the LR73 cells (Figure 5 and data not shown).

Immunostaining for Dock180 revealed that it was predominantly in the cytoplasm when expressed alone, while some Dock180 became localized to membrane proximal regions when cotransfected with ELMO1-GFP (Supplemental Figure S4B, see Supplemental Data, below). Moreover, we could detect a colocalization of ELMO1 and Dock180 (Supplemental Figure S4D). Immu-

rather than engulfed particles (Tosello-Trampont et al., 2001). The average MFI values of duplicate samples calculated from such histograms is indicated on the top of the bar graphs. Where the error bars are not visible, they were too small to be apparent. Other plasmid concentrations,done as part of dose response in the same experiment, gave qualitatively similar results. These data are representative of at least three independent experiments.

(C) Functional cooperation between ELMO1, Dock180, and CrkII. LR73 cells were analyzed in a phagocytosis assay (as described above) after transient transfection with ELMO1-GFP (0.7 μg), Dock180 (1.5 μg), CrkII (0.4 μg), delPSDock180 (1.2 μg), or delGSDock180 (0.8 μg). The average MFI value of duplicate samples is shown at the top of the bar graphs. These data are representative of at least three independent experiments. Where the error bars are not visible, they were too small to be apparent.

(D) Effect of coexpression of Rac1L61 (constitutively active) or Rac1N17 (dominant negative) with ELMO1. LR73 cells were transiently transfected as above with plasmids encoding GFP or ELMO1-GFP (0.8 μg) together with Rac1L61 or Rac1N17 (0.7 μg) and assessed for phagocytosis. The data are representative of at least three independent experiments. Where the error bars are not seen, they were too small to be apparent.

(E) ELMO1/Dock180/CrkII function through Rac during engulfment. LR73 cells were transiently transfected with ELMO1-GFP (0.6 μg), Dock180 (1 μg), CrkII (0.4 μg) and 1.5 μg of the dominant negative Rac1N17. The GFP positive cells were analyzed for phagocytosis as above. The expression of all of the transfected proteins was confirmed by immunoblotting for the individual proteins in total lysates taken from parallel samples. Where the error bars are not seen, they were too small to be apparent.

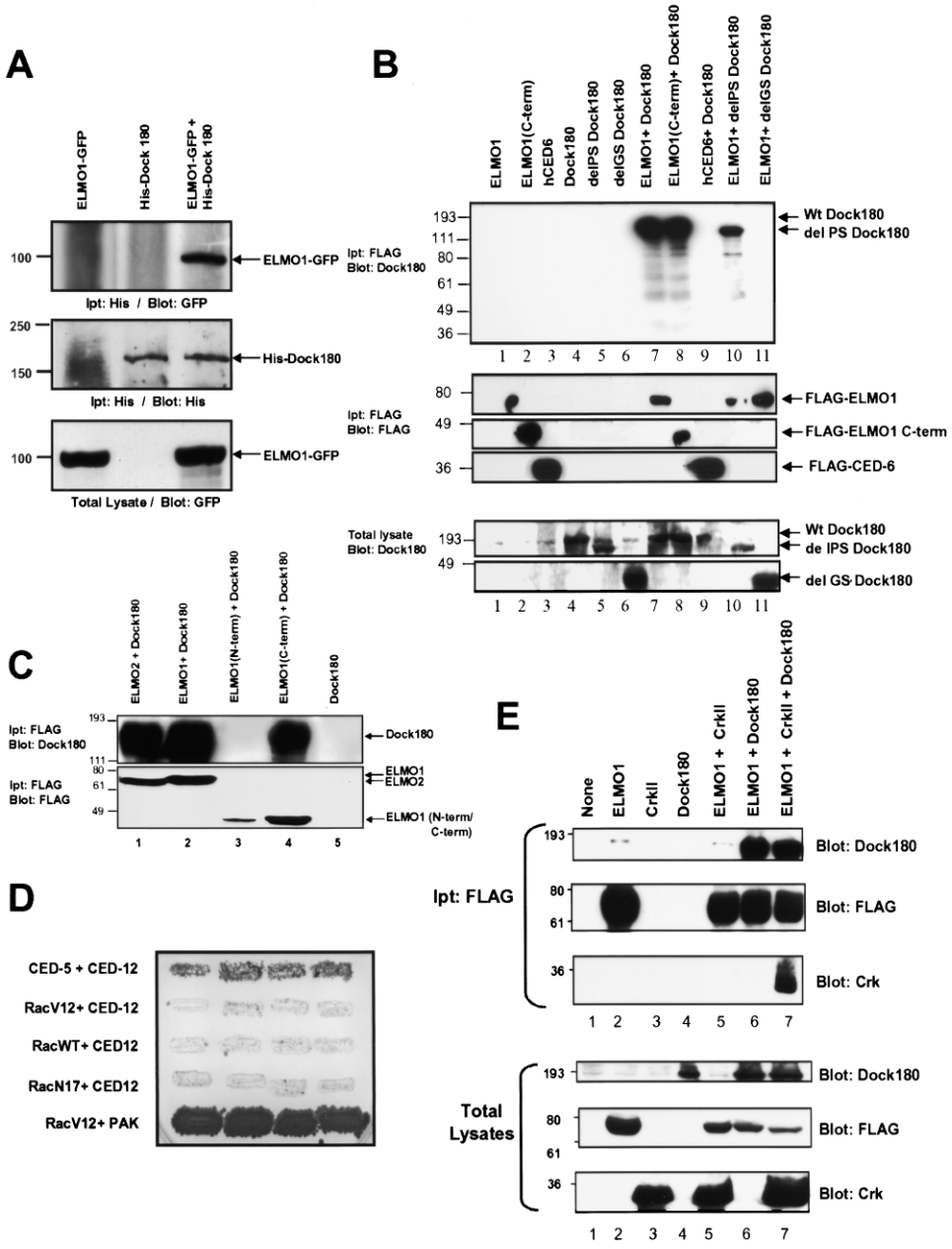


Figure 4. Biochemical Interaction between ELMO1, Dock180, and CrkII

(A) ELMO1-GFP binds Dock180. COS-7 cells were transfected with 5 μg of ELMO1-GFP and His-tagged Dock180 plasmids, either alone or together. After 48 hr, the cells were lysed and immunoprecipitated using anti-His antibodies, and immunoblotted with anti-GFP or anti-His antibodies. The total lysates from the same experiment were immunoblotted with anti-GFP antibody to determine ELMO1-GFP (100 kDa) expression.

(B) N-terminal region of Dock180 is required for interaction with ELMO1. COS-7 cells were transfected with the indicated plasmids, and the lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Dock180 (a mixture of N- and C-terminal antibodies). The same blot was stripped and reprobed with anti-FLAG to determine expression of the FLAG-tagged proteins. Immunoblotting of total lysates indicated the expression of Dock180 in the appropriate lanes.

(C) ELMO1 and ELMO2 interact with Dock180. COS-7 cells were transfected as above with plasmids coding for FLAG-tagged ELMO1, ELMO2, or the N-term or C-term versions of ELMO1 (see schematic in Figure 2C), along with His-Dock180. After anti-FLAG immunoprecipitation, the coprecipitation of Dock180 was analyzed by immunoblotting.

(D) CED-12 interacts with CED-5, but not Rac, in a yeast two-hybrid assay. Interaction between *C. elegans* CED-12 and CED-5, as well as Rac wt and mutants, was analyzed by yeast two-hybrid interaction as described (Supplemental Experimental Procedures). Growth seen on selective plates is shown.

(E) Formation of a trimeric complex between ELMO1, CrkII, and Dock180. After transient transfection of 293T cells with the indicated plasmids, the cells were lysed using Triton X-100, and the lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-CrkII, anti-FLAG, or anti-Dock180. Immunoblotting of the total lysates indicated the expression of the proteins in the appropriate lanes. In this experiment, we could also faintly detect the endogenous Dock180 coprecipitating with ELMO1 (lanes 2 and 5). Data shown in (A)–(E) are representative of at least two to three independent experiments.

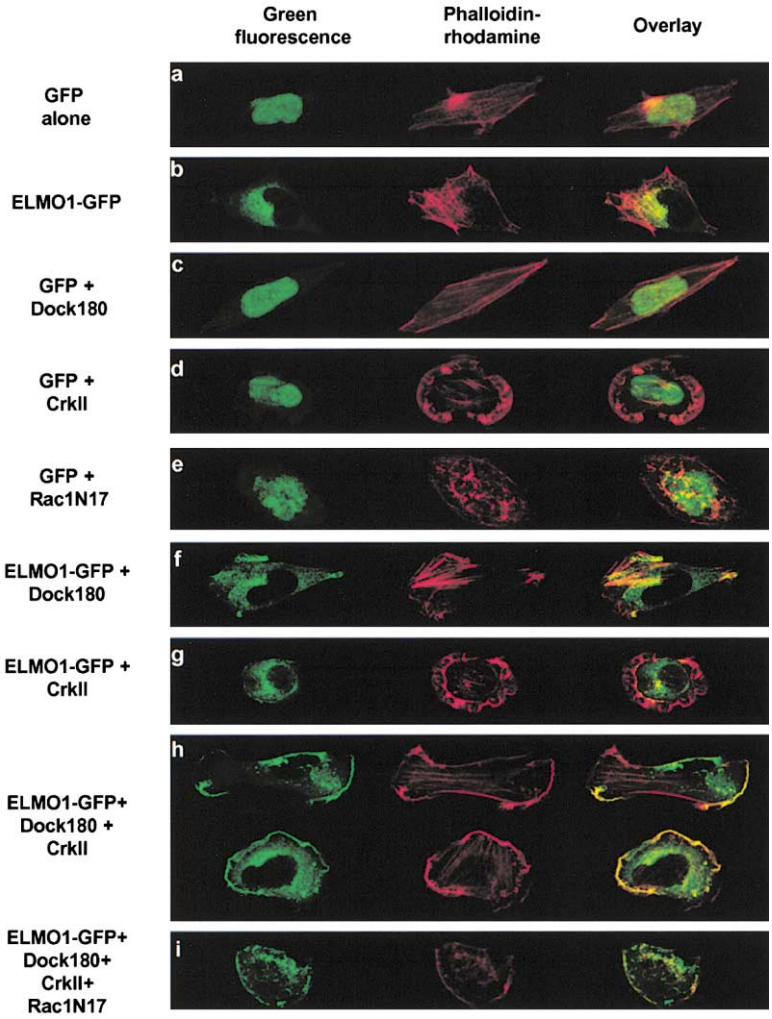


Figure 5. Membrane Localization of ELMO1-GFP

The indicated plasmids were transfected into LR73 cells using Lipofectamine 2000 reagent at the following concentrations: GFP alone (0.4 μg), ELMO1-GFP (0.6 μg), GFP /Dock180 (0.4/1.0 μg), ELMO1-GFP/Dock180 (0.6/1.0 μg), ELMO1-GFP/Dock180/CrkII (0.6/1.0/0.4 μg), and ELMO1-GFP/Dock180/CrkII/Rac1N17 (0.6/1.0/0.4/1.5 μg). 20 hr after transfection, the cells were stained with phalloidin-rhodamine and analyzed by confocal microscopy. The regions of overlay of the green ELMO1-GFP fluorescence with the red fluorescence of phalloidin-rhodamine are represented as yellow. The images shown are representative of multiple cells with similar phenotype on the same slide and are representative of three independent experiments.

nostaining for CrkII also revealed colocalization of CrkII and ELMO1-GFP when these two proteins were coexpressed (Supplemental Figure S4E). When all three proteins were coexpressed, Dock180 and CrkII proteins were readily detectable in membrane ruffles together with ELMO1 (Supplemental Figures S4F and S4G). These data suggest that ELMO1 can localize to membrane ruffles through a process that is influenced by Dock180 and CrkII proteins.

ELMO1 Associates with a Rac-GEF Activity, but Is Unlikely To Be the GEF Itself

To test whether ELMO1 could associate with a Rac guanine nucleotide exchange factor (GEF) activity, 293T cells were transfected with either tagged ELMO1 alone, Dock180 alone, or cotransfected with both ELMO1 and Dock180. The cell lysates were immunoprecipitated with antibodies to the tagged ELMO or Dock180, and the presence of GEF activity toward Rac was assessed. While precipitates from ELMO-1 alone expressing cells had little GEF activity, there was a readily detectable Rac-GEF activity found in ELMO-1 precipitates from cells coexpressing ELMO1 and Dock180 (Figure 6A). Direct Dock180 precipitates also had detectable and reproducible Rac-GEF activity, suggesting that either Dock180 itself or another protein closely associated with Dock180 could be the GEF. A bacterially produced and purified GST-tagged ELMO-1 had no detectable Rac-GEF activity in the same assay (Figure 6A).

We also tested whether ELMO1 overexpression can promote Rac-GTP loading within cells (using the Rac-GTP pull-down through the CRIB domain of PAK). Overexpression of ELMO1 alone in 293T cells did not lead to Rac-GTP loading, while ELMO-1 was able to augment the Dock180 dependent Rac-GTP loading (Figure 6C). Taken together, these data suggested that while ELMO1 itself is most likely not the GEF, it could associate with a GEF activity within cells and regulate Rac-GTP loading.

Discussion

Genes that regulate engulfment of apoptotic cells in *C. elegans* were first identified almost twenty years ago (Hedgecock et al., 1983; Ellis et al., 1991). The recent cloning of six of the seven genes implicated in engulfment of cell corpses and the existence of the mammalian orthologs of these genes have provided an exciting opportunity to begin to understand the molecular

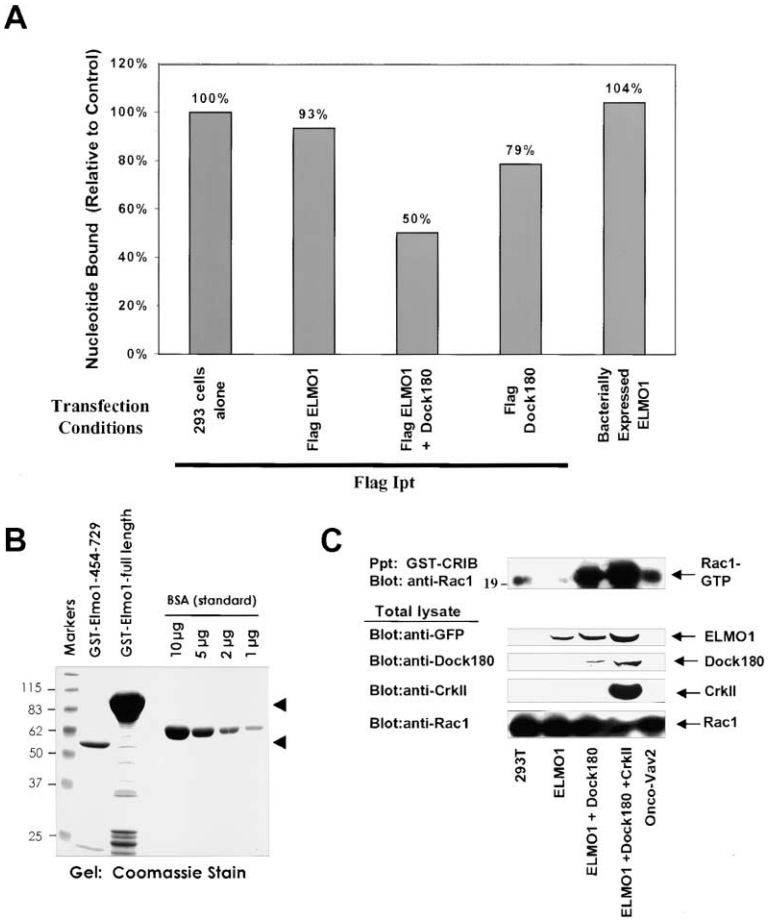


Figure 6. A Rac-GEF Activity Is Associated with ELMO1

(A and B) 293T cells were transfected with the indicated plasmids, and the cell lysates were immunoprecipitated with anti-Flag antibody. The proteins bound to the beads were analyzed for GEF activity toward Rac by incubation with α^{32}P-GTP loaded Rac (bacterially produced and purified) in a buffer containing an excess of unlabeled GTP as described (Nemergut et al., 2001). The presence of GEF-activity was revealed by loss of radioactivity bound to Rac (i.e., due to the exchange reaction). The ^{32}P-GTP bound to Rac when incubated with precipitates from untransfected 293T cells (which was identical to incubation with exchange buffer alone) was set at 100%. Expression of bacterially produced full-length ELMO-1, as well as a shorter control fragment (454–729), was confirmed (B). These data are representative of 2–3 independent experiments. Similar results were observed in an assay measuring the loading of labeled GTP onto Rac.

(C) Coexpression of ELMO1 and Dock180 promotes Rac-GTP loading in cells. 293T cells were transiently transfected with plasmids encoding the indicated proteins. The lysates from transfected cells were then precipitated using bacterially produced CRIB-domain of PAK. The Rac-GTP that was precipitated was visualized by immunoblotting for Rac. The total lysates from this experiment were immunoblotted for the transfected proteins. The expression of Dock180 alone led to Rac-GTP loading, but less than coexpression of ELMO+Dock180, but higher than the background (data not shown).

details of this important biological process. Here, we have identified the seventh gene, *ced-12* in *C. elegans*, as well as its orthologs in *Drosophila*, human, and mouse. Several lines of data suggest a role for CED-12 and ELMO proteins in phagocytosis and cell migration, and place CED-12/ELMO, together with CED-2/CrkII and CED-5/Dock180, at a step upstream of activation of CED-10/Rac1. Since mutations in *ced-12* cause multiple defects in the worm, we predict that CED-12 and its mammalian orthologs play a key role in integrating signals from a variety of stimuli to Rac dependent actin-based cytoskeletal rearrangements.

Genetically, *ced-2/ced-5/ced-10* and *ced-1/ced-6/ced-7* segregate into two partially redundant groups (Ellis et al., 1991). All *ced-12* alleles that we analyzed showed a defect in both engulfment and cell migration, similar to those observed in *ced-2*, *ced-5*, and *ced-10* mutant animals (Reddien and Horvitz, 2000; Wu and Horvitz, 1998b). Double mutant analyses also placed *ced-12* in the same group as *ced-2/ced-5/ced-10*. Since none of these four genes in this complementation group encodes a receptor, the upstream activators of the *ced-2/ced-5/ced-10/ced-12* pathway for engulfment remain elusive (Liu and Hengartner, 1998; Reddien and Horvitz, 2000; Wu and Horvitz, 1998a, 1998b; Zhou et al., 2001). In mammals, a number of receptors that participate in engulfment have been recognized, including some integrins that are known to function upstream of Crk (Savill and Fadok, 2000). To test whether integrins might participate in cell corpse recognition, we have looked at known mutants in the two *C. elegans* α integrins, pat-2 and ina-1, as well as in the single *C. elegans* β integrin, pat-3. None of the mutants showed any evidence of persistent cell corpses (Supplemental Table S2, see Supplemental Data). Although this does not rule out a role for integrins, it strongly argues against their involvement as upstream regulators of the CED-2/CED-5/CED-12/CED-10 cell corpse engulfment pathway.

The fact that the DTC migration defect can still be observed in *ced-12:ced-3* double mutants where persistent cell corpses are not seen (Tables 1 and 2) suggests that the engulfing cells and migrating cells may receive different cues. Whether any of the genes implicated in DTC migration might encode upstream regulators of *ced-2/ced-5/ced-12/ced-10* remains to be tested (Montell, 1999; Nishiwaki, 1999).

CED-12/ELMO and CED-5/Dock180 Connection

We observed an evolutionarily conserved interaction between CED-12/ELMO and CED-5/Dock180. Since ELMO1 binds to a mutant of Dock180 that lacks the CrkII binding sites, and CED-12 and CED-5 could interact in the absence of CED-2 in yeast two-hybrid assays (there is no Crk II homolog in *S. cerevisiae*), our working hypothesis is that an ELMO1:Dock180 complex might exist initially, to which CrkII would bind. However, CrkII could

still strengthen the ELMO:Dock180 interaction in a trimeric complex. Dock180 is a large protein of 1866 amino acids, and contains an SH3 domain at its extreme N terminus and proline-rich motifs near its C terminus (Hasegawa et al., 1996), but other functional domains have not yet been fully characterized. Our preliminary studies suggest that the SH3 domain of Dock180 can interact with the PXXP motif at the C terminus of ELMO1 (Supplemental Figure S5). Further studies are required to determine possible additional contacts between these proteins, the ELMO/Dock180 interaction at endogenous levels of expression, and the stoichiometry of these proteins in a complex (Kiyokawa et al., 1998b). So far, our antibodies generated against ELMO have not been of high enough affinity to address these questions (data not shown). A second isoform of Dock180, Dock-2, with a more restricted expression in hematopoietic cells, as well as other less well-characterized homologs of Dock180, have been described (Nishihara et al., 1999). The potential interaction of Dock-2 or other Dock180 homologs with the different ELMO proteins remains to be tested.

The precise reason for partial inhibition of phagocytosis seen with either ELMO alone or Dock180 alone expression remains unclear; however, the enhancement of uptake seen when the two proteins are expressed suggests that perhaps the ELMO/Dock180 complex needs to be at a certain optimum level, and that overexpression of one component may affect the balance. Although the most efficient uptake in the phagocytosis assays was seen when all three proteins were cotransfected, the synergy observed after cotransfection of either ELMO1 and Dock180 or ELMO and CrkII was likely mediated through the endogenous Dock180 or CrkII interacting with the transfected proteins.

CED-12/ELMO1 and Rac Activation

Based on several lines of evidence presented here, we favor a model whereby ELMO, in conjunction with Dock180 and CrkII, would lead to Rac1 activation. Our working model for Rac activation through the ELMO1/Dock180/CrkII pathway is as follows. We hypothesize that ELMO1 and Dock180 might be in a complex initially, and resides predominantly in the cytoplasm. Since CrkII is not generally found in complex with Dock180 under basal conditions (Kiyokawa et al., 1998b; our unpublished data), CrkII may not be part of the ELMO/Dock180 complex. Either the "eat-me" signals on apoptotic cells or migration cues would promote an interaction of CrkII with Dock180, and, in turn, with ELMO. It is not known where the trimeric complex is formed and whether one or more than one of these three proteins mediates the interaction with a putative receptor(s) on the membrane. It is noteworthy that the ELMO1-CAAX construct did not provide a gain of function for ELMO1, suggesting another level of regulation besides the simple membrane localization. Once assembled, the trimeric complex likely recruits a GEF and promotes the GTP/GDP exchange on the nucleotide-free Rac that is bound to Dock180. Consistent with this model, we observe that ELMO1 precipitates, when cotransfected with Dock180, possess a Rac-GEF activity in vitro. Dock180 has been shown to bind nucleotide-free Rac, a conformation of Rac that appears to be favored by guanine nucleotide exchange factors (GEFs) (Erickson et al., 1997; Kiyokawa et al., 1998a; Nolan et al., 1998). Although we observed some Rac-GEF activity in Dock180 precipitates, Dock180 itself does not possess an obvious GEF-like domain in its primary sequence and is thought to recruit a Rac-GEF (Hasegawa et al., 1996; Kiyokawa et al., 1998a). Although we considered the possibility that CED-12/ELMO might be the GEF, our sequence analyses failed to detect any Rac GEF domain in CED-12/ELMO polypeptides. Moreover, bacterially expressed, purified ELMO proteins failed to show Rac GEF activity in vitro. Thus, it appears more likely that an existing or yet to be identified Rac-GEF activity is associated with the ELMO1/Dock180/CrkII complex and leads to Rac activation. Given the various defects seen in *ced-12* mutant worms, it is likely that CED-12/ELMO itself recruits a Rac-GEF or regulates the nucleotide exchange on Rac in some other critical way. Other proteins implicated in cell migration might also be recruited to the CrkII/Dock180/ELMO complex and could regulate Rac-dependent signaling. Testing the validity of this model is currently in progress.

Experimental Procedures

Mutations and Strains

All mutant animals used in these studies were derived from the wild-type variety Bristol strain N2. SNP mapping was performed with wild-type variety Hawaii strain CB4856. The following mutations have been described (Hodgkin et al., 1988), except for the *ced-12* alleles, which were described by Chung et al. (2000) or in this study: LG I: *dpy-5(e61)*, *unc-29(e1072am)*, *mec-8(e398am)*, *ced-12(oz167)*, *ced-12(bz187)*, *ced-12(k145)*; *ced-12(k149)*; *ced-12(k156)*; *ced-12(k158)*; *lin-11(e566)*, *unc-75(e950)*, *ced-1(e1735)*. LG III: *ced-6(n1813)*, *ncl-1(e1865)*, *ced-7(n1892)*. LG IV: *ced-2(e1752)*, *ced-5(n1812)*, *ced-10(n1993)*, *him-8(e1489)*, *ced-3(n717)*. LG V: *him-5(e1490)*. The ced-12 allele *oz167* was isolated in an EMS screen for mutations that affect gonadal morphology (R.F. and T.S., unpublished data); *k145*, *k149*, *k156*, and *k158* were independently isolated in a similar screen (Nishiwaki, 1999). Isolation of the *bz187* mutation was described in Chung et al. (2000). Mapping of *ced-12* through SNPs and Northern analyses were performed as described in Supplemental Experimental Procedures (see Supplemental Data, below).

Analysis and Quantification of Engulfment in *C. elegans*

The appearance and number of cell corpses were analyzed as described previously (Avery and Horvitz, 1987; Sulston and Horvitz, 1977). Cell corpses in the head were scored just before hatching (three-fold stage) or just after hatching (four-cell gonad L1 stage; Klass et al., 1976). Germ cell corpses were assayed 24 to 36 hr after the L4 molt to adulthood. The average corpse numbers and the standard error of the mean (SEM) were then determined.

Germline Transformation and Genomic Rescue of *ced-12*

Transgenic animals were generated by germline microinjection, using the YACs Y45G3, Y49H10, or Y39F7 at 10 to 40 ng/μl into adult *ced-12(oz167)* hermaphrodites with the coinjection marker pRF4 at 80 ng/μl. pRF4 carries the mutated collagen gene *rol-6(su1006gf)* and confers a dominant roller (Rol) phenotype. Transgenic lines carrying stably transmitting extrachromosomal arrays were kept and assayed for persistent cell corpses. Rescue of *ced-12(oz167)* was considered to have occurred in lines where most late-stage embryos had four or fewer head cell corpses.

Transgenic Rescue Experiments

Full-length *ced-12* cDNAs or flag-tagged *elmo1* or *elmo2* were cloned into modified heat shock promoter vectors pPD49.78 and

pPD49.83 (Mello and Fire, 1995). We overexpressed *ced-12* or *elmo* either before the embryonic cell deaths occurred or after (at L1 and L2 stages) by heat shock at 33°C for 20–30 min. The expression of the transgene and corpses in the head region were then scored (see Supplemental Experimental Procedures for more details).

Cloning of *elmo1* and *elmo2*

Two consensus primers representing *elmo1* sequences were designed (based on mouse and human ESTs) and used in RT-PCR of polyA$^+$ RNA from RAW264.7 mouse macrophage line and in PCR amplification of pACT human placental library (provided by Steve Elledge, Dallas) and a human macrophage library. After confirmation of the amplified sequence as *elmo1*, nested primers for human *elmo1* and vector arms of the pACT library were designed and used in PCR to obtain the 5′ end of *elmo1*. After subcloning of the PCR products, multiple clones were sequenced. The sequence of human *elmo2*, mouse *elmo1*, and mouse *elmo2* were obtained by sequencing the EST clones BEO17955, AI574349, and AA711524, respectively. The human *elmo2* EST clone BEO17955 contains an intron sequence between nucleotides 120–187 (numbered beginning with ATG); this was determined by computer analyses of other human *elmo2* ESTs and comparison to the human genome sequence and the mouse ELMO2 protein sequence. The coding sequences of the mouse ELMO1 and ELMO2 were subcloned into the pEBB vector (Tanaka et al., 1995), with a C-terminal FLAG tag or an EGFP tag (Clontech). The C-term and N-term versions of ELMO1 (carrying deletions of amino acids 18–325 or 332–620, respectively) were generated by digesting the pEBB-ELMO1 with PflMI and BstBI or BstBI and NdeI enzymes, respectively, and adding a linker to maintain the coding frame. Plasmids encoding full-length Dock180, delPS and delGS mutants, Dock-CAAX, wt CrkII, and CrkIIW169L were kindly provided by Michiyuki Matsuda (Kiyokawa et al., 1998b).

In Vitro Phagocytosis Assays

Maintenance of the phagocytic LR73 Chinese hamster ovary (CHO) cell line and transient transfections of LR73 in 24-well plates have been described previously (Tosello-Trampont et al., 2001). When cotransfecting multiple plasmids, carrier DNA was added to match plasmid concentration in the different samples. 20 hr posttransfection, the cells were incubated (in serum-free medium for 2 hr) with 2 μm or 0.1 μm carboxylate-modified red fluorescent beads (Sigma Chemical Co.), indicative of phagocytosis and pinocytosis, respectively. After washing, the cells on the plate were trypsinized and resuspended in cold medium with 1% sodium azide, and 30,000 cells were analyzed for each point by two-color flow cytometry (Tosello-Trampont et al., 2001). Forward and side-scatter parameters were used to distinguish the unbound beads from cells. The transfected cells were recognized by their GFP fluorescence (Tosello-Trampont et al., 2001). The engulfment assays were performed at 20 hr posttransfection, when the ELMO1 transfected cells appeared morphologically similar to cells expressing GFP alone (data not shown).

Immunoprecipitations, Immunoblotting and Confocal Microscopy

See Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data can be found at the *Cell* website at http://www.cell.com/cgi/content/full/107/1/27/DC1.

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Accession Numbers

GenBank Accession Numbers for the human and mouse elmo1 and elmo2 sequences are AF398883, AF398884, AF398885, and AF398886.